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FEBS Letters 347 (1994) 203–206

FEBS 14158

The crystal structures of reduced pseudoazurin from *Alcaligenes faecalis* S-6 at two pH values

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Received 25 April 1994

Abstract

The structures of the reduced (Cu^{1+}) blue-copper protein pseudoazurin from *Alcaligenes faecalis* strain S-6 are refined at pH 7.8 and 4.4 using X-ray diffraction data to 1.8 Å resolution. The final *R*-factors for the high and low pH structures are 0.178 and 0.177, respectively. Comparing the reduced pseudoazurin at pH 7.8 with the oxidised (Cu^{2+}) molecule, small changes are observed in the vicinity of the copper site and on the protein surface. At pH 4.4 the copper substituent imidazole of His^{81} rotates away from the metal with a concurrent movement of the latter towards the plane of the remaining three ligands ($S\gamma$-Cys^{78}, $N\delta1$-His^{40} and $S\delta$-Met^{86}) thus the geometry of the copper site becomes planar trigonal.

Key words: Blue copper protein; Crystal structure; pH effect; Pseudoazurin; Redox state; *Alcaligenes faecalis*

1. Introduction

Pseudoazurin is a soluble periplasmic redox protein (123 amino acid residues; MW 13397 Da) participating in the electron transport pathway leading to the reduction of NO_2^- to mainly NO, and to a lesser extent to N_2O, in the potent denitrifying bacterium *A. faecalis* [1]. The in vivo electron donor to pseudoazurin is unknown to date. Its electron acceptor is believed to be the green copper protein nitrite reductase [2] whose structure is also under investigation in our laboratory. The structure of the oxidised pseudoazurin at pH 6.8 has been refined at 1.55 (Å) resolution [3]. A closely related blue copper protein, the poplar plastocyanin, has been investigated by X-ray structure analysis in both the oxidised and reduced states at several pH values [4]. A major pH effect had been observed on the structure of the reduced plastocyanin; the imidazole ring of the Cu-ligand His^{87} rotates 180° around the $C\beta$–$C\gamma$ bond at pH 3.8 thus leaving the Cu^{1+} ion co-ordinated with the remaining three ligands. This crystallographic result directly supported the results of studies carried out in solution, i.e. the pH-dependent electron transfer rate constants between inorganic oxidants and plastocyanin, and pH-dependent values of the standard reduction potential [5,6]. From these investigations it was expected that a protonated redox inactive form of plastocyanin would be dominant at pH < 5.4. Similar studies have been carried out recently for pseudoazurin of *Achromobacter cycloclastes* [7], a protein with 66% identity to the one studied in the present communication. In these studies, a pH effect on the kinetics of reduced pseudoazurin with inorganic oxidants as well as a pH-dependent redox potential were also observed. Finally, an analogous pH-dependent change of the reduced structure has been observed for amicyanin, another member of the blue copper protein family, as was deduced from solution ^1H NMR studies on amicyanin from *Thiobacillus versutus* [8].

In order to investigate the structure and function relationship for pseudoazurin from *A. faecalis*, we determined the co-ordination of the metal ion in the reduced state of the protein at two distant pH values (4.4 and 7.8). The pH was varied in order to be able to observe any protonation effects on the imidazole groups of the metal-binding histidine residues.

2. Experimental

The protein was extracted from transformed *E. coli* JM 105 with the recombinant plasmid pUB1 in which the pseudoazurin gene is under the control of both *lac* and *tac* promoters [9] and the ampicillin resistance gene is present. The recombinant pUB1 plasmid was kindly provided by Prof. T. Beppu (University of Tokyo). The bacteria were grown in 2× YT medium containing 1 mM $CuSO_4$ and 50 μg/ml ampicillin. The induction was done with 1 mM IPTG when the optical density of the cell suspension reaches A_{600nm} = 0.5 and the growth continues until A_{600nm} = 1.6. The cells from 1 liter culture were resuspended in 10 ml of 10 mM Tris-HCl buffer pH 7.5 and disrupted by sonication. Before and after the sonication protease inhibitors were added: 1 mM PMSF, 42 μM leupeptin and 1 mM benzamidine. The cell lysate was centrifuged at 14,000 rpm for 30 min and the supernatant was applied to a DEAE-Sephacel (Pharmacia) anion-exchange column equilibrated with 10 mM Tris-HCl pH 7.5. The flow-through fractions were dialysed against 20 mM NaH_2PO_4/K_2HPO_4 pH = 6.8 containing 40 mM KCl.

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Abbreviations: CM, carboxymethyl; DEAE, diethylaminoethyl; IPTG, isopropyl-β-D-thiogalactoside; PMSF, phenylmethylsulfonylfluoride; rms, root-mean-square; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)amino-methane.

The coordinates of the reduced pseudoazurins will be deposited with the Protein Data Bank, Brookhaven National Laboratory, Upton, NY, USA.

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SSDI 0014-5793(94)00544-6

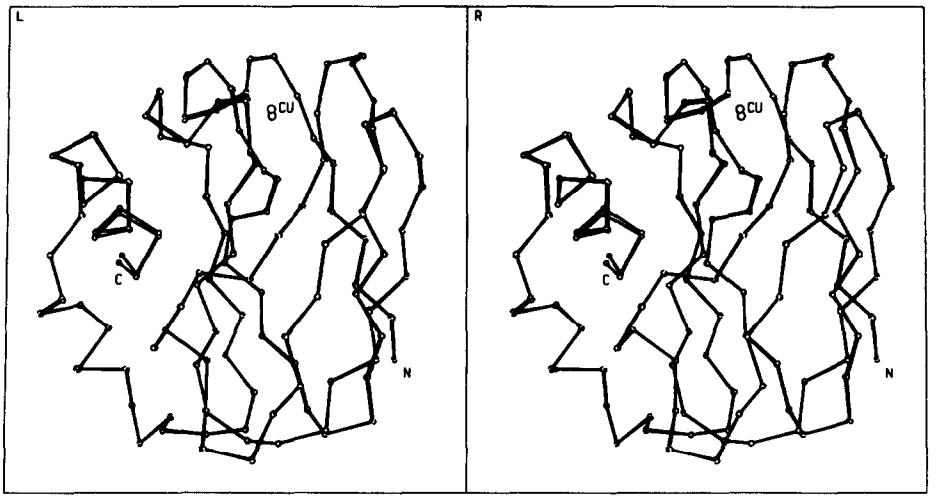

Fig. 1. Stereo diagramme of the Cα-atoms of the superimposed oxidised (thin lines) and reduced pseudoazurin at pH 4.4 (thick lines). The amino- and carboxy-termini as well the metal site are labeled.

The dialysate was in turn applied to a CM-Sepharose CL-6B (Pharmacia) cation exchange column equilibrated at pH 6.8 with the same buffer. A linear KCl gradient (40 mM to 500 mM) was applied and the fractions eluted at 130–240 mM KCl were collected, pooled and dialysed extensively against 50 mM CH_3COONH_4 pH 5.7. The protein solution was concentrated first in a Centriprep-10 concentrator unit and secondly in a Centricon-10 microconcentrator unit (Amicon) to a concentration of about 6 mg of protein/ml. 2.4 g of cell pellet usually yields 7.8 mg of essentially pure pseudoazurin as estimated from the absorbance ratio A_{593nm}/A_{277nm} = 0.43 and SDS-PAGE.

The protein was crystallised in its oxidised state as described earlier [10] except that 50 mM CH_3COONH_4 buffer pH 5.7 was used instead of 50 mM KH_2PO_4/K_2HPO_4 buffer pH 8.0. The average size of the crystals obtained was 0.4 mm × 0.6 mm × 1.0 mm. In order to gradually shift the pH of the crystals, we immersed them in a series of buffered 3.3 M $(NH_4)_2SO_4$ solutions. Their pH values differed by 0.2–0.3 of the pH unit. The crystals were left to equilibrate in each buffer for about 2 h. Finally, we immersed the crystals in 3.3 M $(NH_4)_2SO_4$ solutions buffered with 50 mM Na-citrate pH 3.8 and 100 mM Tris-HCl pH 7.8. The reduction was carried out by adding solid Na-ascorbate (final concentration 100 mM) and was followed by the bleaching of the deep blue colour of the crystals. The corresponding pH values measured after the addition of Na-ascorbate were 4.4 and 7.8. The symmetry of the reduced white crystals and the unit cell parameters remained essentially the same as for the oxidised blue crystals [3,10]. The use of the image plate detector-scanner system from MARresearch (Hendrix, J. and Lentfer, A., unpublished result) in combination with the short wavelength radiation (λ = 0.92 Å) of the synchrotron beam-line X11 (EMBL at DESY, Hamburg) made possible the collection of two 98% complete data sets (10.–1.8 Å resolution). A total of 67,800 and 97,396 Bragg reflections were collected from the high and low pH crystals in about 7 and 11 h respectively. After processing of the images with the modified programmes of the MOSCO package [11] we obtained 12,660 independent reflections for each data set with a merging R-factor of 0.05 ($R_{merge} = \Sigma|I_i - \langle I \rangle|/\Sigma I_i$). The refined model of the oxidised pseudoazurin served as the starting point for the restrained least-squares refinement [12]. 300 and 210 cycles were carried out using the programme SFALL [13] for the high and low pH structures respectively. The details of the refinement procedure will be published elsewhere. All computations were done using programmes of the CCP4 suite [13] and manual interventions used the interactive molecular graphics programme FRODO [14] on an Evans and Sutherland PS390 system.

3. Results and discussion

As in the oxidised pseudoazurin the three residues at the C-terminus could not be modeled as there was not enough electron density to define any limited number of conformations. For the final refined models the crystallographic reliability indexes (R-factors, where

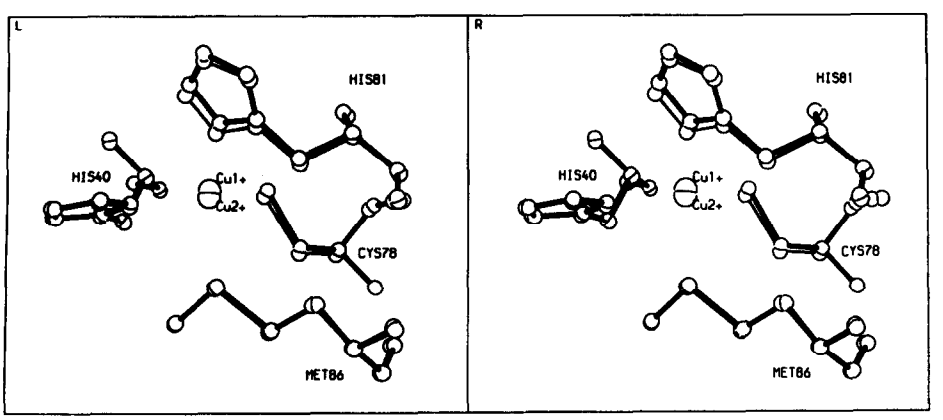

Fig. 2. The copper-site of oxidised (thin lines) and reduced pseudoazurin at pH 7.8 (thick lines). The metal ions and the ligand residues are labeled.

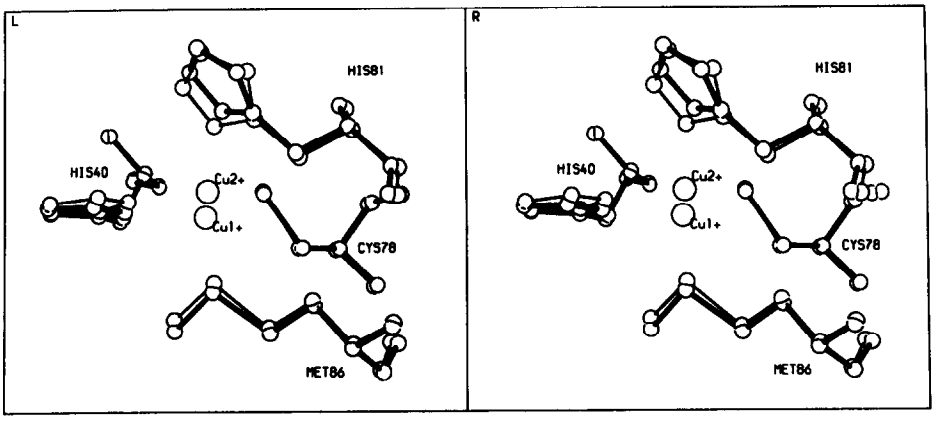

Fig. 3. The copper-site of oxidised (thin lines) and reduced pseudoazurin at pH 4.4 (thick lines). The metal ions and the ligand residues are labeled.

R-factor = $\Sigma|F_{obs}-F_{calc}|/\Sigma F_{obs}$) including all 12,660 Bragg reflections in the 10.–1.8 Å resolution range were: 0.178 for pH 7.8 and 0.177 for pH 4.4 and, the rms-deviations from ideality for bond-lengths and angles were 0.017 Å and 3.2° respectively. The final models include 918 protein atoms and, 98 or 94 ordered water molecules for the high pH or the low pH structure, respectively. The mean temperature factors for the high and low pH structures were 26.9 Å^2 and 29.1 Å^2. The rms-displacement of the main chain atoms was 0.12 Å (max. = 0.47 Å) for oxidised pseudoazurin and the pH 7.8 reduced structure. The corresponding deviation for the reduced structure at pH 4.4 was 0.18 Å (max. = 0.92 Å). The stereo diagramme of the Cα-atoms of the superimposed coordinates (Fig. 1) of the oxidised and the low pH reduced structures shows how small are the changes upon reduction with regard to the main chain fold. The changes at the blue copper site upon reduction at high and low pH are shown in Figs. 2 and 3, respectively. In the pH 7.8 reduced structure the copper ion moves 0.22 Å and follows the imidazole of His^{81} which rotates by 14° around its Cβ–Cγ bond. On the protein surface, the main chain atoms of: Pro^{35}, Glu^{54}, Asn^{63}, Ile^{119} and Ala^{120} move by 0.3 Å with respect to their position in the oxidised pseudoazurin. In the pH 4.4 reduced structure the copper ion moves 0.69 Å mainly toward its ligand atom Sδ of Met^{86} and the imidazole of His^{81} rotates by 26° around its Cβ–Cγ bond. The metal moves in the opposite direction with respect to its movement in the pH 7.8 reduced structure. The movements at the copper site resemble those in plastocyanin but the extent of the rotation of the second histidine ligand is quite different in these two cases [4]. Some relevant parameters for the active site geometry are listed in Table 1. For the low pH structure the distance of Cu^{1+} to Nδ1-His^{81} is 3.1 Å and the distance to the imidazole-His^{81} plane is 1.55 Å. These results unambiguously indicate that the side-chain of His^{81} no longer is a copper-ligand. A difference Fourier map [($|F_{obs}|-|F_{calc}|$), φ] was computed with calculated structure factor amplitudes (F_{calc}) and phases (φ) derived from the oxidised 'blue' model and observed amplitudes (F_{obs}) from the low-pH reduced 'white' crystal. Part of this electron-density map and the copper-site of the oxidised state of the protein is shown in Fig. 4. In this map

Table 1
The geometry of the Cu-site in the reduced pseudoazurin

	Distances Å			Angles (°)			Distance of Cu^{1+} from planes (Å)	
	pH 7.8[a]	pH 4.4[b]		pH 7.8[a]	pH 4.4[b]		pH 7.8[a]	pH 4.4[b]
Cu–40Nδ1	2.16	2.19	40Nδ1–Cu–78Sγ	140	138	Imidazole ring of His^{40}	0.07	0.09
Cu–78Sγ	2.17	2.16	40Nδ1–Cu–81Nδ1	102	94	Imidazole ring of His^{81}	0.30	1.55
Cu–81Nδ1	2.29	3.09	40Nδ1–Cu–86Sδ	85	97	40Nδ1, 78Sγ, 81Nδ1	0.37	0.77
Cu–86Sδ	2.91	2.42	78Sγ–Cu–81Nδ1	108	83	40Nδ1, 78Sγ, 86Sδ	0.63	0.22
Cu–39O	3.83	4.01	78Sγ–Cu–86Sδ	107	122	40Nδ1, 81Nδ1, 86Sδ	1.13	1.09
78Sγ–41N	3.75	3.72	81Nδ1–Cu–86Sδ	110	116	81Nδ1, 78Sγ, 86Sδ	0.84	0.87
40Nε2–9Oδ1	2.84	2.96	Cu–78Sγ–78Cβ	105	96			
81Nε2–Ow	2.69	2.88						

[a]reduced structure at pH 7.8
[b]reduced structure at pH 4.4

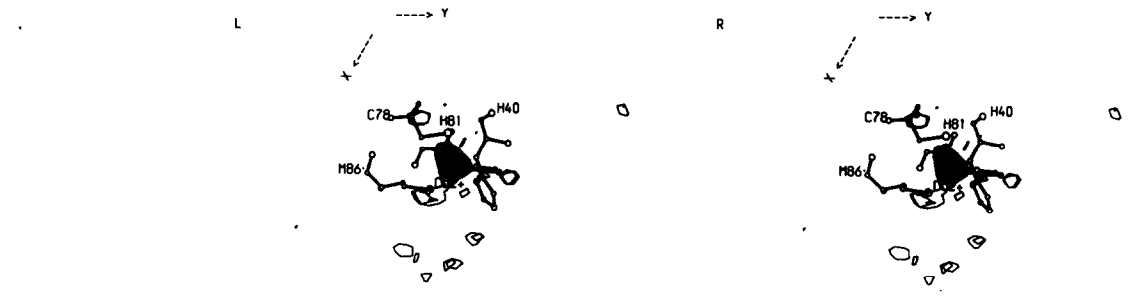

Fig. 4. Difference Fourier (F_o-F_c) map computed with phases from the oxidised pseudoazurin, and F_o from the reduced crystals at pH 4.4. The copper and its ligands of the oxidised molecule are also shown. Contouring levels of the map start at 0.3 e/Å^3 and are incremented by 0.2 e/Å^3.

it is shown clearly that the major change occurs near the copper. Its movement toward Sδ-Met^{86} is dictated unequivocally by the diffraction data. The Cu^{1+} ion lies very close (0.2 Å) to the plane defined by the ligand atoms (Sγ-Cys^{78}, Nδ1-His^{40} and Sδ-Met^{86}). The direction of the third histidine of the protein (His^6) which points to the solvent region seems to be unaffected by the reduction of the metal and the pH change.

The general conclusion that is to be drawn from these results is that pseudoazurin is an efficient redox protein at high pH values with small structural changes occuring around the metal-site upon reduction as is expected for an electron transfer protein obeying the outer sphere mechanism [15]. On the other hand, at low pH, the redox active form of the protein is in equilibrium with a protonated redox inactive form whereby the Cu^{1+} assumes a stable planar trigonal geometry [16]. It has been recently established that for the homologous pseudoazurin from *A. cycloclastes* the pK_a of His^{81} is 4.9 [7]. It could be safely deduced from the latter work and the present structural study that the critical pH value for the change of redox activity for pseudoazurins should be 4.9 which is near to the one for plastocyanins (4.8) [6] and lower than the corresponding value for amicyanins (6.8) [8].

Acknowledgements: The authors would like to thank Prof. Teruhiko Beppu for providing the recombinant plasmid pUB1. The travel to the synchrotron radiation source was supported by the Large Installations Project Programme from the European Union.

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